Intersectin, a Novel Adaptor Protein with Two Eps15 Homology and Five Src Homology 3 Domains*

(Received for publication, May 14, 1998, and in revised form, September 2, 1998)

Montarop Yamabhai‡§, Noah G. Hoffman§, Nancy L. Hardison¶, Peter S. McPherson‖**, Luisa Castagnoli‡‡, Gianni Cesareni‡‡§§, and Brian K. Kay‡¶¶

From the ‡Department of Pharmacology, University of Wisconsin, Madison, Wisconsin 53706-1532, the §University of North Carolina, Chapel Hill, North Carolina 27599-3280, the ¶Duke University Medical Center, Durham, North Carolina 27710, the ‖Department of Neurology and Neurosurgery, Montreal Neurological Institute, McGill University, Montreal, Quebec, H3A 2B4, Canada, and the ‡‡Department of Biology, Enrico Calef, University of Rome Tor Vergata, Via Della Ricerca Scientifica, 00173 Rome, Italy

We screened a *Xenopus laevis* oocyte cDNA expression library with a Src homology 3 (SH3) class II peptide ligand and identified a 1270-amino acid-long protein containing two Eps15 homology (EH) domains, a central coiled-coil region, and five SH3 domains. We named this protein Intersectin, because it potentially brings together EH and SH3 domain-binding proteins into a macromolecular complex. The ligand preference of the EH domains were deduced to be asparajine-proline-phenylalanine (NPF) or cyclized NPF (CX_{1-2}NPFXXC), depending on the type of phage-displayed combinatorial peptide library used. Screens of a mouse embryo cDNA library with the EH domains of Intersectin yielded clones for the Rev-associated binding/Rev-interacting protein (RAB/Rip) and two novel proteins, which we named Intersectin-binding proteins (Ibps) 1 and 2. All three proteins contain internal and C-terminal NPF peptide sequences, and Ibp1 and Ibp2 also contain putative clathrin-binding sites. Deletion of the C-terminal sequence, NPFL-COOH, from RAB/Rip eliminated EH domain binding, whereas fusion of the same peptide sequence to glutathione S-transferase generated strong binding to the EH domains of Intersectin. Several experiments support the conclusion that the free carboxylate group contributes to binding of the NPFL motif at the C terminus of RAB/Rip to the EH domains of Intersectin. Finally, affinity selection experiments with the SH3 domains of Intersectin identified two endocytic proteins, dynamin and synaptojanin, as potential interacting proteins. We propose that Intersectin is a component of the endocytic machinery.

The EH[1] domain has recently been described as a protein interaction module involved in endocytosis (1). The domain was first discovered in Eps15, a tyrosine kinase phosphorylation substrate of the epidermal growth factor receptor (2). Eps15 is a ~145,000 Da protein with three EH repeats and has been shown to be a component of endocytic vesicle intermediates (3–5). Biochemical analysis of the Eps15 EH domains have shown that they are likely involved in protein-protein interactions: far-Western blotting and affinity chromatography experiments demonstrate that a number of cellular proteins can bind to the Eps15 EH fusion protein (2), and recently, several potential cellular ligands have been identified (6). Within the *Saccharomyces cerevisiae* genome there are five EH domain-containing proteins, two of which, Pan1 and End3, have been shown to have roles in endocytosis (7–9).

Another protein interaction module is the Src homology 3 (SH3) domain. This domain is 50–70 amino acids long and is present in numerous signal transduction and cytoskeletal proteins (10, 11). Examination of the ligand specificity of SH3 domains has revealed that they recognize proline-rich sequences containing the core peptide sequence PXXP (12, 13). SH3 domains have proposed roles in directing the assembly of NADPH oxidase subunits (14), modulating the activity of phosphatidylinositol 3′-kinase (15) and the GTPase activity of dynamin (16), as well as localizing proteins to distinct subcellular sites (17).

Interactions between SH3 domains and PXXP sequence-containing proteins have also been shown to contribute to synaptic vesicle endocytosis. For example, amphiphysin I, amphiphysin II, and endophilin are SH3 domain-containing proteins that are enriched in the nerve terminal and that interact through their SH3 domains with proline-rich sequences in dynamin and synaptojanin (18–25). An interesting connection between SH3 domain- and EH domain-mediated interactions has been revealed by the observation that the 170-kDa alternative splice variant of synaptojanin (19, 26) interacts with the EH domains of Eps15 (27, 28), as well as with endophilin and the amphiphysins.

During the course of our investigations into SH3 domains and peptide ligands, we found that we could screen cDNA expression libraries with synthetic peptide ligands of SH3 domains and isolate cDNAs encoding SH3 domain-containing proteins (29). We have screened cDNA expression libraries of a 16-day-old mouse embryo, human prostate, and human hematopoietic cells and identified 18 different SH3 domain contain-

[1] The abbreviations used are: EH, Eps15 homology; AP, alkaline phosphatase; COLT, cloning of ligand targets; Dap, dynamin-associated protein; GST, glutathione S-transferase; Ibp, Intersectin-binding protein; MP90, mitotic phosphoprotein of 90 kDa; RAB, Rev-associated binding protein; Rip, Rev interacting protein; SH3, Src homology 3; PAGE, polyacrylamide gel electrophoresis.

ing cDNAs, 10 of which are novel. We have named this approach cloning of ligand targets (COLT) (29). In this report, we describe the structure and molecular recognition properties of a novel protein isolated in a COLT screen of a *Xenopus laevis* oocyte cDNA library. We have named this protein Intersectin, because it potentially brings several proteins together into a macromolecular complex through its two EH and five SH3 domains. Evidence is presented in support of the interaction of Intersectin with two protein components of the endocytic machinery, dynamin and synaptojanin, as well as several novel putative clathrin-binding proteins.

MATERIALS AND METHODS

Isolation of Frog Intersectin cDNA Clones—A *Xenopus laevis* oocyte cDNA λzapII library (courtesy of Dr. Alan Wolffe, National Institutes of Health) was screened by COLT (29) with a biotinylated Src SH3 class II binding peptide, biotin-SGSGILAPPVPPRNTR-NH$_2$. A single binding isolate was recovered from a screen of ~10^4 plaques, rescued (30), and sequenced. The insert was used to screen a *X. laevis* tadpole cDNA λzapII library (courtesy of Dr. Doug DeSimone, University of Virginia) by molecular hybridization. Positive clones were rescued and sequenced, to generate a full-length coding region.

Determination of the Peptide Ligand Preferences of the EH Domains of Intersectin—Glutathione *S*-transferase (GST) fusions to the individual EH domains of Intersectin were generated by amplifying the appropriate regions from the cDNA sequence by polymerase chain reaction (31) and subcloning them into pGEX2TK (Amersham Pharmacia Biotech). GST fusion protein was purified according to the manufacturer's instructions. The peptide ligand preferences of both EH domains were determined by screening (32) two different bacteriophage M13 libraries: an X_9 library displayed on the major (pVIII) capsid protein (33), and an X_{10}C library displayed on a minor (pIII) capsid protein (33). Each combinatorial peptide library contained over 200 million clones, where *X* is any amino acid encoded by NN(G/T) codons. The primary structures of the peptides displayed by binding phage were determined by DNA sequencing.

Screening cDNA Libraries with EH-alkaline Phosphatase Fusion Protein—A λEXlox mouse 16-day-embryo cDNA expression library (Novagen, Madison, WI) was screened according to a published protocol (34). Plasmids bearing the cDNA inserts were rescued from the isolated λ phage by Cre-mediated excision, and the inserts were sequenced by fluorescent dideoxynucleotide sequencing.

Microtiter Plate Assays for Peptide Binding to EH Domains—Various peptide sequences were fused genetically to the N terminus of secreted alkaline phosphatase (AP) using published methods (34). Microtiter plate wells, coated with approximately 1 μg of GST fusions to EH domains of Intersectin, End3p, or Pan1p, were incubated with different peptide-AP fusions for 1 h. After washing, the amount of enzyme retained in the wells is measured with a colorimetric assay using *p*-nitrophenyl phosphate and a microtiter plate spectrophotometer (Molecular Devices, Sunnyvale, CA) at a wavelength of 405 nm.

To evaluate the relative binding strength of various peptides for the EHa domain, a competition assay was used. Microtiter plate wells, coated with approximately 2 μg of GST fused to EHa, were incubated with an AP fusion to the peptide sequence DCTNPFRSCWR, along with individual peptides at varying concentrations. The following synthetic peptides were purchased (Research Genetics, Huntsville, AL): NPF1 (Ac-PSTNPFVAA-CONH$_2$), NPF2 (Ac-SSTNPFWTN-CONH$_2$), NPF3 (Ac-VSSNPFMTG-CONH$_2$), NPFL-COOH (Ac-GSSSTNPFL-COOH), NPFL-CONH$_2$ (Ac-GSSSTNPFL-CONH$_2$), and cyclized NPF (biotin-GSGSDCTNPFRSCWR-CONH$_2$; intramolecular disulfide bond). The peptides were >75% pure, and their identities were confirmed by mass spectroscopy.

Pull-down Experiments with Protein Extracts from Brain—Adult rat brains were homogenized at 1:10 (weight:volume) in 20 mM HEPES-OH (pH 7.4) containing 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, and 0.5 μg/ml leupeptin followed by centrifugation for 5 min at 800 × g_{max}. The resulting postnuclear supernatants were incubated with 1% Triton X-100 for 1 h at 4 °C, and insoluble material was removed by centrifugation at 180,000 × g_{max} for 1 h. The soluble supernatant (1 mg of protein) was incubated overnight at 4 °C with various GST fusion proteins (~25 μg of each fusion protein) coupled to glutathione-Sepharose. Samples were then washed extensively in 20 mM HEPES-OH (pH 7.4) containing 0.83 mM benzamidine, 0.23 mM phenylmethylsulfonyl fluoride, 0.5 μg/ml aprotinin, 0.5 μg/ml leupeptin, and 1% Triton X-100; bound proteins were then eluted with

SDS-PAGE sample buffer. The samples were then processed for Western blot analysis with antibodies against dynamin (18), synaptojanin$_{145}$ (35), or synaptojanin$_{170}$ (19).

RESULTS

In a COLT screen (29) of a *Xenopus laevis* oocyte cDNA expression library with a Src SH3 class II peptide ligand probe, we isolated a single reactive cDNA clone. Upon sequencing, we determined that the clone encoded a segment of a protein with five SH3 domains at its C terminus. To isolate the full-length version of this mRNA, we probed a tadpole cDNA library by nucleic acid hybridization and recovered several full-length and overlapping clones. Fig. 1*A* portrays the amino acid sequence of the protein that we have named Intersectin, to suggest its possible role as an adaptor or scaffold molecule. The 1270-amino acid-long protein (predicted molecular mass, 143,581 Da) has two EH domains, a central coiled-coil region, and five C-terminal SH3 domains (Fig. 1*B*). The two Intersectin EH domains are 38% identical to each other and ~45% similar to EH domains identified in the genomes of yeast, nematode, and mouse.

BLAST searches of GenBank^{TM} show Intersectin to be a novel protein, with the greatest similarity to two human (SH3P17 and SH3P18) and two fly proteins (Dap160−1 and Dap160−2). Although SH3P17 and SH3P18 protein sequences are not full-length (29), they share 84–85% amino acid identity with Intersectin, with the highest level of matching between the SH3 domains (98% for SH3P17 and 90% for SH3P18). The fly proteins, referred to as dynamin-associated proteins (Daps), Dap160−1 and Dap160−2, are alternatively spliced products that are very similar to Intersectin, except that they contain four instead of five SH3 domains (36). Even though the human cDNA clones are incomplete, we propose that Intersectin, SH3P17, SH3P18, and Dap160 are encoded by homologous genes or members of a gene family in frogs, humans, and flies.

Based on earlier experiments demonstrating that EH domains of Eps15 and Eps15R could react with numerous proteins in far Western blots (2), we hypothesized that the Intersectin EH domains would have the capacity to interact with short peptide ligands. Therefore, each EH domain was subcloned into pGEX2T to generate GST fusions that were used to screen phage-displayed combinatorial peptide libraries (37–39). Fig. 2, *A* and *B,* lists the amino acid sequences of phage that were affinity selected from the 9-mer library (33) with the EHa and EHb domains of Intersectin, respectively. Both domains selected peptides with the tripeptide consensus, asparagine-proline-phenylalanine (NPF), although phage selected with the EHa domain tended to bind better to EHa than EHb, and *vice versa*. Interestingly, NPF was also identified in affinity selection experiments with the EH domains of Eps15 and Eps15R (6), suggesting that the EH domains of Intersectin, Eps15, and Eps15R have similar or equivalent peptide specificity.

We next performed affinity selection experiments with a second combinatorial peptide library, displayed on the minor capsid protein pIII. We isolated three different peptides with the NPF motif; however, in this case, the NPF sequences were flanked on each side by cysteines (Fig. 2*C*). The presence of the cysteines suggested to us that conformational constraint, provided by intramolecular disulfide bonds, may enhance the binding of the NPF tripeptide to the Intersectin EH domains. To explore this possibility, we fused different peptide sequences to a secreted form of the *Escherichia coli* AP protein (34). Fusion of the sequence DCTNPFSCWR to the N terminus of AP yielded a protein capable of binding strongly to microtiter plate wells coated with GST fusions to the EH domains of Intersectin, Eps15, but not of the yeast proteins End3p and Pan1p (Fig.

A

```
    1  MAQFGTPFGG NLDIWAITVE ERAKHDQQFH GLKPTAGYIT GDQARNFFLQ SGLPQPVLAQ   60
   61  IWALADMNND GRMDQLEFSI AMKLIKLKLQ GYPLPSILPS NMLKQPVAMP AAAVAGFGMS  120
  121  GIVGIPPLAA VAPVPMPSIP VVGMSPPLVS SVPTVPPLSN GAPAVIQSHP AFAHSATLPK  180
  181  SSSFGRSVAG SQINTKLQKA QSFDVPAPPL VVEWAVPSSS RLKYRQLFNS QDKTMSGNLT  240
  241  GPQARTILMQ SSLPQSQLAT IWNLSDIDQD GKLTAEEFIL AMHLIDVAMS GQPLPPILPP  300
  301  EYIPPSFRRV RSGSGLSIMS SVSVDQRLPE EPEEEEPQNA DKKLPVTFED KKRENFERGN  360
  361  LELEKRRQAL LEQQRKEQER LAQLERAEQE RKERERQDQE RKRQQDLEKQ LEKQRELERQ  420
  421  REEERRKEIE RREAAKRELE RQRQLEWERN RRQELLNQRN REQEDIVVLK AKKKTLEFEL  480
  481  EALNDKKHQL EGKLQDIRCR LTTQRHEIES TNKSRELRIA EITHLQQQLQ ESQQLLGKMI  540
  541  PEKQSLIDQL KQVQQNSLHR DSLLTLKRAL ETKEIGRQQL RDQLDEVEKE TRAKLQEIDV  600
  601  FNNQLKELRE LYNKQQFQKQ QDFETEKIKQ KELERKTSEL DKLKEEDKRR MLEQDKLWQD  660
  661  RVKQEEERYK FQDEEKEKRE ESVQKCEVEK KPEIQEKPNK PFHQPPEPGK LGGQIPWMNT  720
  721  EKAPLTINQG DVKVVYYRAL YPFDARSHDE ITIEPGDIIM VDESQTGEPG WLGGELKGKT  780
  781  GWFPANYAER MPESEFPSTT KPAAETTAKP TVHVAPSPVA PAAFTNTSTN SNNWADFSST  840
  841  WPTNNTDKVE SDNWDTWAAQ PSLTVPSAGQ HRQRSAFTPA TVTGSSPSPV LGQGEKVEGL  900
  901  QAQALYPWRA KKDNHLNFNK NDVITVLEQQ DMWWFGEVQG QKGWFPKSYV KLISGPLRKS  960
  961  TSIDSTSSES PASLKRVSSP AFKPAIQGEE YISMYTYESN EQGDLTFQQG DLIVVIKKDG 1020
 1021  DWWTGTVGEK TGVFPSNYVR PKDSEAAGSG GKTGSLGKKP EIAQVIASYA ATAPEQLTLA 1080
 1081  PGQLILIRKK NPGGWWEGEL QARGKKRQIG WFPANYVKLL SPGTNKSTPT EPPKPTSLPP 1140
 1141  TCQVIGMYDY IAQNDDELAF SKGQVINVLN KEDPDWWKGE LNGHVGLFPS NYVKLTTDMD 1200
 1201  PSQQFRLGVK PAGGIPATGD RPFILFPFRD GPSLLPNAFQ APPLSVVMIK FRCFTAPRFC 1260
 1261  PDMNVKYINI     1270
```

B

FIG. 1. **Amino acid sequence (*A*) and schematic diagram of *Xenopus laevis* Intersectin (*B*).** Amino acid sequences of two EH (EHa and EHb) and five SH3 (SH3A, SH3B, SH3C, SH3D, and SH3E) domains are *underlined* and represented as *boxes* in the diagram. The middle of the protein contains a putative coiled-coil region (52) that may form a dimer with itself or other proteins. The GenBank accession number of Intersectin is AF032118.

3). When the same peptide sequence was fused to AP, but with serine replacing the cysteines, binding was abolished. These results demonstrate that the structural conformation of NPF-bearing peptides, provided by intramolecular disulfide bonds, contributes to binding. This conclusion is supported by the inability of two peptide ligands, isolated from the pVIII library (Fig. 2), to bind the EH domains when fused to AP (Fig. 3).

Two reasons may potentially account for the apparent differences in the selection outcomes from the pVIII and pIII phage-displayed libraries. First, due to the higher display valency of the major capsid protein (2500 copies *versus* 5), weak interactions may be preserved during selection due to avidity effects. Second, the major capsid protein may have contributed a structural conformation to the NPF peptide, optimizing its binding to the Intersectin EH domains. The major coat protein adopts a near-crystalline state (40, 41), which influences the structure of attached peptides (42).

To learn what proteins may interact with the EH domains of Intersectin, we initially screened the frog oocyte cDNA expression library with the two EH domains of Intersectin, fused separately to bacterial AP (34). Unfortunately, this screen proved negative. However, screening a 16-day mouse embryo cDNA library, which we have used extensively (29), yielded four reactive clones (Fig. 4*A*). They comprised three different, incomplete sequences: two were novel, and the other was identified as the murine homolog of the Rev-associated binding/Rev-interacting protein (RAB/Rip) (43, 44). We have named the two novel mouse proteins Intersectin-binding proteins (Ibps), Ibp1 and Ibp2. All three predicted proteins carried three to four copies of the tripeptide NPF, agreeing with the outcome of the

experiments with the combinatorial peptides. Interestingly, not only is the spacing of the NPF motifs comparable between the three proteins, but they also shared similar C-terminal sequences (*i.e.* TNPFL-COOH or TNPFLL-COOH). Although both EH domains of Intersectin interact with the three proteins, in general the EHa domain binds more strongly than EHb.

Sequence analysis of Ibp1 and Ibp2 revealed a number of interesting features. First, both proteins have a 66-amino acid segment of primary structural similarity that includes two of the internal NPF motifs (Fig. 4, *double-underlined residues*). This segment is also present in MP90 (Fig. 4*B*), a mitotic phosphoprotein (45), and a nematode protein of unknown function, T040C10.2 (not shown). Second, within the 66-amino acid segment is the sequence LV(D/N)LD (Fig. 4, *italicized*), which resembles the consensus sequence L(L/I)(D/E/N)(L/F)(D/E), recently delineated for clathrin binding (46–48). Third, Ibp1 has a 25-amino acid segment in common with MP90 (Fig. 4, *dotted underlining*). Within this segment is the tripeptide DPW, which is present two, three, and nine times in Ibp1, Ibp2, and MP90, respectively. It should also be noted, that in the EH domain-containing protein, Eps15 (49), which has been shown to be involved in endocytosis (3, 28, 50, 51), there are 10 copies of a similar tripeptide sequence, DPF. The superficial similarity of the DPW and DPF motifs to NPF suggests that they may also serve as ligand sites for a protein interaction module(s). Fourth, an 81-amino acid segment (Fig. 4, A and B, *single-underlined*) is shared between Ibp2 and MP90. Examination of GenBank has revealed that this 81-amino acid segment is part of a larger (*i.e.* ~140 amino acids) conserved element, present

A

```
   QS NPF GNAT
   EK NPF VDAT
  GRS NPF MRT
  SSI NPF LRT
    T NPF SSAG
   SK NPF GGVA
    T NPF DAPIA
    N NPF QAAR
  RAT NPF GFA  *
    S NPF GNPAA
   RH NPF IERA
GRSAR NPF L
    K NPF GPVPS
```

B

```
  TSK NPF AE
RAHIN NPF M
LWSST NPF A
   PS NPF VTQ
 RYAN NPF Q
  EST NPF LRW  *
      NPF VTAYAR
  KSH NPF LAP
      NPF YTSQTP
RKISN NPF S
      NPF ADKLPQ
RLRSF NPF
```

C

```
VECGW NPF VSC
AVFCS NPF LAC
  DCT NPF RSCWR  *
```

FIG. 2. **Amino acid sequence alignment of Intersectin EH domain binding peptides isolated from two M13 bacteriophage-displayed combinatorial peptide libraries.** Amino acid sequences of peptides selected for binding to EH domain a (*A*) and b (*B*) from a library of 9-mer peptides displayed on protein VIII. Panel *C* indicates peptide sequences from a screen of an X_5FX_5 and $X_{10}C$ peptide library displayed on protein III that bind to both EHa and EHb; the fixed cysteine residue is *underlined*. All phages were isolated once, except for the isolate displaying DCTNPFRSCWR, which was isolated five times. * indicates sequences fused to AP.

FIG. 3. **Binding characteristic of selected Intersectin EH domain-peptide ligand-AP fusions.** Various GST fusion proteins and Anti-FLAG monoclonal antibody (*Anti-FLAG*) were immobilized in triplicate wells of microtiter plate and incubated with different peptide-AP fusions. Bound AP fusion proteins retained in the wells after 1 h of incubation were detected by colorimetric assay with *p*-nitrophenyl phosphate. The sequence of the peptides fused to the N terminus of AP are shown at the *top* of each histogram. To demonstrate that equal amounts of protein were immobilized onto the wells, they were incubated with a polyclonal antibody to GST (Amersham Pharmacia Biotech), followed by detection with a secondary antibody conjugated to horseradish peroxidase and 2′,2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid. The average absorbances at a wavelength of 405 nm and S.D. are shown.

at the N terminus of at least 12 proteins.[2] The function of all of these proteins with the ~140-amino acid conserved element is unknown, except that there is some weak similarity to a region within the clathrin assembly protein AP180 (53). The colinear similarity between the Ibp1, Ibp2, MP90, and T040C10.2 proteins suggests that they are likely related in cellular function.

To evaluate whether the internal or C-terminal NPF repeats within the RAB/Rip protein were responsible for binding to the EHa domain, a simple truncation experiment was performed. As seen in Fig. 5*A*, although EHa bound to GST-RAB/Rip when blotted to a membrane, it failed to bind to GST- RAB/Rip when it lacked 5 amino acids (TNPFL) at its C terminus. Conversely, binding was robust when TNPFL was fused to the C terminus of GST. Because the binding of the EHa-AP to the GST-RAB/Rip and GST-TNPFL fusions appeared equivalent, we hypothesized that the EH domains of Intersectin interact principally with the C-terminal TNPFL sequence within RAB/Rip.

This hypothesis was tested with synthetic peptides. Peptides corresponding to the four NPF motifs of RAB/Rip were tested for their ability to compete the binding of an AP fusion protein to the EHa domain of Intersectin. Fig. 6 demonstrates that the IC_{50} value for the C-terminal NPFL-COOH peptide of RAB/Rip is 40 μM, whereas the IC_{50} values for the three internal motifs are 600–1000 μM. Thus, the C-terminal peptide sequence of RAB/Rip is a much better ligand than its internal peptides, even though they are all the same length (*i.e.* 9-mers). The
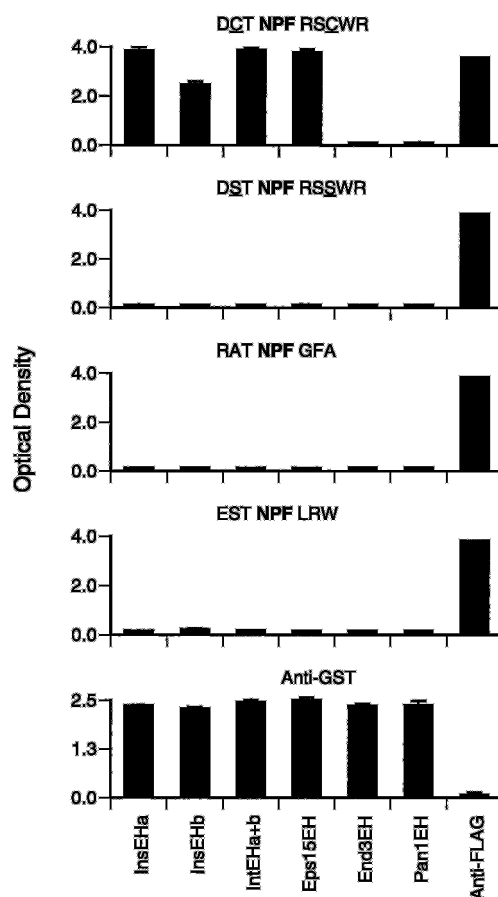
importance of the carboxylate group in the C-terminal NPFL-COOH peptide is underscored by the observation that same sequence, but with a carboxamide instead of a carboxylate group, has an IC_{50} value of 300 μM. Finally, by comparison a cyclized NPF peptide, isolated from a phage-displayed combinatorial peptide library (Fig. 2*C, asterisk*), is the best ligand, with an approximate IC_{50} of 3 μM. Similar results were obtained with the EHb domain (data not shown).

When the NPFLX_{0-1}-COOH motif was used to search GenBank, we came across the MP90 protein (Fig. 4*B*). This protein, with an apparent molecular mass of 90,000 Da, was originally identified in a screen for *X. laevis* mitotic phosphoproteins (45). To confirm that MP90 potentially interacted with the EH domains of Intersectin, we tested whether or not MP90 could be selected from a mixture of two labeled components. Fig. 5*B* demonstrates that GST fusions to either EHa or EHb, but not Src SH3, could bind specifically to MP90.

Based on the occurrence of the NPF motif at the C terminus of the RAB, Ibp1, Ibp2, and MP90 proteins, we wondered how close the motif needed to be at the C terminus to bind well to the Intersectin EHa domain. In Fig. 7, the NPF motif is successively displaced from the C terminus of a fusion to GST by

---

A

Mouse RAB/Rip

SANADFANFDAFGQSSGSSNFGGFPTASHSSFQPQTTGGSAGSV
NANFAHFDNFPKSSSADFGTFSTSQSHQTASTVSKVSTNKAGLQ
TADKYAALANLDNIFSAGQGGDQGSGFGTTGKAPVGSVVSVPSH
SSASSDKYAALAELDSVFSSAATSSNAYTPTSNASSSVFGTVPV
GASAQTQPASSGPAPFGATPST**NPF**VAATGPSAASST**NPF**QTN
ARGATAATFGTASMSMPAGFGTPAQYSLPTSFSGSFQQPAFPAQ
AAFPQQTAFSQQPNGAGFATFGQTKPVVTPFGQVAAAGVSS**NPF**
MTGAPTGQLPTGSSST**NPFL**

Ibp1

SSSDPWAPAPAFSDPWGGSPAKPSSNGTAAVGGFDTEPDEFSDF
DRLRTALPTSGSSTGELELLAGEVPARSPGAFDMSGVGGSLAES
VGSPPPAATPTPTPPPTRKTPESFLGPNAA*LVDLDS*LVSRPGPTP
PGSKAS**NPF**LPSGAPPTGPSVT**NPF**QPAPPATLTLNQLRLSPV
PPVPGAPPTYISPLGGGPGLPPMMPPGPPAPNT**NPF**LL

Ibp2

SSKALTLLDYLIKTGSERVAQQCRENIFAIQTLKDFQYIDRDGK
DQGINVREKSKQLVALLKDEERLKVERVQALKTKERMAQVATGV
GSNQITFGRGSSQPNLSTSYSEQEYGKAGGSPASYHGSTSPRVS
SELEQARPQTSGEEELQLQLALAMSREVAEQEERLRRGDDLRLQ
MALEESRRDTVKVPKKKEAKACCKPGSHSQQTTLLDLMDALPSS
GPVTQKTEPWSAGASANQTNPWGGTVAPSNITDPWPSFGTKPAA
SVDPWGVPTTASTQSVPKNSDPWAASQQPASNAGKTTDAWGAAK
PSSASGGSFELFSNFNGTVKDDFSEFDNLRTSKKPAESGASVPPQ
DSRTTSPDLFESQSLTSASSKPSSARKTPESFLGPNAA*LVNLDS*
LVTKPAPPAQSL**NPF**LAPGAAAPAPVN**PF**QVNQPQPLTLNQLR
GSPVLGSSASFGSGPGVETVAPMTSVAPHSSVGASGSSLTPLGP
TAMNMVGSVGIPPSAAQSTGTT**NPF**LL

B MP90

MKNIVHNYSEAEIKVREATSNDPWGPSSSLMSEIADLTYNVVAF
SEIMSMIWKRLNDHGKNWRHVYKAMTLMEYLIKTGSERVAQQCK
ENIYAIQTLKDFQYVDRDGKDQGVNVREKAKQLVSLLKDDERLK
EERAHALKTKEKLAQTSTSSSASSTLNPAPEGEQAWSQSSGEEE
LQLQLALAMSKEEAEQVRAKPPPVSEEELQLQLALSLSKEEHDK
EERIKRGDDLRLQMALEESRKGAPSKQEEQSSLMDLDADVFSPPA
PVAPTADPWGASAAPPADPWAGGATPASVPAAAAAPDPWGGPPV
ATGSSSDPWGTGVQTNSTPGDPWGGTQAVTSADVKSVSDPWNPG
GSGATTAIPSDPWSSSPPVAQSVKKAADPWAPPAASFSDPWGGS
PSKPNTNGTMGELDLLAGEVPMSRSLGSKSPDAFDMSTMSGSLC
DFSNPTRKTPESFLGPNAA*LVDLDS*LISKSTLQNTKTS**NPF**LVT
GTPNPTAT**NPF**QPNQQSSLTLNQLRSSPVMTLGQPVTPAGQTPA
TIPFASPMMSVSPMAPGIPLANMAPMVGMQPMAGVPVGTLAPGV
PGMVLPPMMPPQQLVAQPLLPNLSTQAVT**NPF**L

FIG. 4. **Amino acid sequences of Intersectin EH domain-interacting proteins.** *Panel A* illustrates the primary structures of four cDNA clones isolated from a 16-day mouse λ-cDNA expression library probed with a mixture of EHa- and EHb-AP fusions. Three different protein sequences were identified: the mouse homolog of the RAB/Rip (43, 44), and two novel sequences, Ibp1 (isolated twice) and Ibp2. *Panel B* indicates the amino acid sequence of the *Xenopus laevis* MP90 protein (45), which was identified in a computer search of GenBank with the NPFLX_{0-1}-COOH motif. Polypeptide segments shared between the different proteins are *single-underlined* (81-amino acid-long segment in both Ibp2 and MP90) and *double-underlined* (66-amino acid-long segment in Ibp1, Ibp2, and MP90). NPF residues are in *boldface*. Putative clathrin-binding sites are in *italics*. The GenBank accession numbers for mouse RAB/Rip, Ibp1, and Ibp2 are AF057287, AF057285, and AF057286, respectively.

the insertion of variable numbers of alanine residues. Although there is strong binding to GST-TNPFL, binding is reduced with the insertion of one alanine and absent with the insertion of two or more alanines at the C terminus. Thus, even though the fusion proteins are present in equivalent amounts, binding by the Intersectin EHa domain requires that the motif be NPFL-COOH or NPFL*X*-COOH. Future experiments will address the precise requirements of the ligand for a free carboxylate group, now that the three-dimensional structure of one EH domain has been determined (54).

To characterize the protein-protein interaction specificity of the SH3 domains of Intersectin, combinatorial peptide libraries
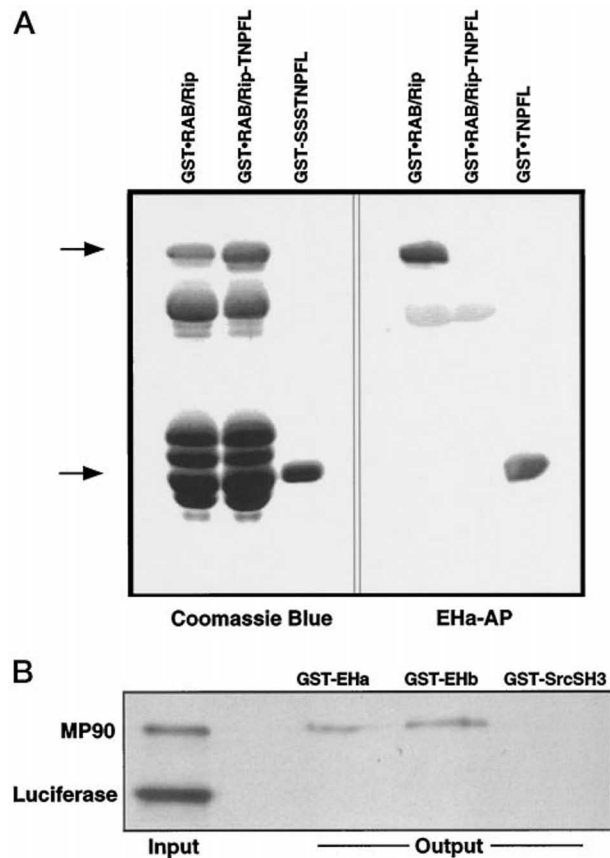
A

B

FIG. 5. *In vitro* **binding of EH domain interacting proteins.** *Panel A* demonstrates that TNPFL motif at the C terminus of RAB/Rip is responsible for its interaction with EHa domain of Intersectin. GST fusions to the isolated RAB cDNA clone (GST·RAB), a truncated form of RAB from which the TNPFL sequence was deleted (GST·RAB-TNPFL), and the TNPFL motif (GST·TNPFL) were resolved by 12% SDS-PAGE and blotted to polyvinylidene difluoride membrane. EH-interacting proteins were detected on the membrane (*right panel*) with the Intersectin EHa fused to AP (EHa-AP) and NBT/BCIP. Proteins were stained in a parallel gel (*left panel*) with Coomassie Brilliant Blue. *Panel B* demonstrates the specific interaction between MP90 and Intersectin EH domains. MP90 and luciferase proteins were generated *in vitro* with coupled transcription-translation in the presence of tRNA charged with biotinylated lysine. The protein mixture was chromatographed with GST fusions to Intersectin EHa, EHb, and Src SH3 immobilized on glutathione-agarose. Input and output samples were resolved by 12% SDS-PAGE and blotted to polyvinylidene difluoride membrane for detection with streptavidin-HRP and a chemiluminescent substrate.

were screened with the SH3A domain.[3] When the peptide sequences were used to search GenBank by computer, we found related sequences in the guanine nucleotide exchange factor for Ras, Son-of-sevenless, and dynamin. Thus, we decided to determine whether SH3A and the other SH3 domains of Intersectin would affinity select dynamin from rat brains, which are a rich source of well characterized endocytic proteins. As seen in Fig. 8, the SH3A, B, C, and E domains of Intersectin could individually, and quantitatively, recover dynamin and the 145-kDa isoform of synaptojanin (synaptojanin$_{145}$). The 170-kDa isoform of synaptojanin (synaptojanin$_{170}$) could be recovered by only the SH3A, C, and E domains. Because the recovery of these proteins from the rat brain extracts could be due to either direct or indirect interactions, the five SH3 domains were used in far-Western blotting experiments of the extracts. The SH3A domain bound synaptojanin$_{145}$ and dynamin strongly, whereas weak binding was evident for SH3C and SH3E domains, and no binding was evident for the SH3B and SH3D domains (data not

---

[3] B. K. Kay, N. G. Hoffman, and N. L. Hardison, unpublished results.
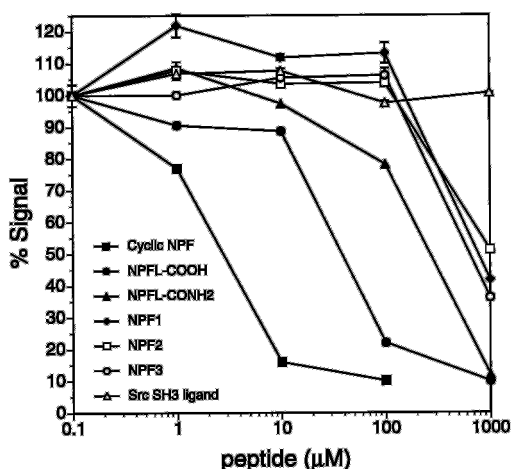
FIG. 6. **Competition of synthetic peptides with the binding of an AP fusion to the EHa domain of Intersectin.** Microtiter plate wells, coated with approximately 2 μg of GST fusion protein to EHa, were incubated with a peptide (DCTNPFRSCWR)-AP fusion and various competitor peptides (μM). After 1 h of incubation, the AP fusion retained in the wells after washing was detected by a colorimetric assay with *p*-nitrophenyl phosphate. *% Signal* corresponds to the average of triplicate samples, normalized to the absorbance (405 nm) obtained in the absence of any soluble peptide; S.D. values are shown. NPF1, NPF2, NPF3, and NPFL-COOH peptides correspond to the three internal NPF motifs (amino acids 201–209, 215–223, and 310–318) and C terminus (amino acids 327–335) of RAB/Rip (Fig. 4). Cyclic NPF and Src ligand peptides correspond to DCTNPFRSCWR (with an intramolecular disulfide bond) and SGSGILAPPVPPRNTR, respectively. Similar results were obtained with the EHb domain (data not shown).
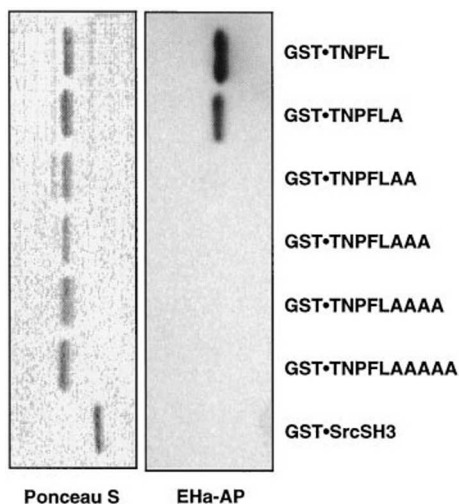
FIG. 7. **The NPF motif must be close to the C terminus of GST to bind well to the Intersectin EHa domain.** A series of peptide fusions, TNPFL(A)$_{1-5}$, fused to the C terminus of GST, were resolved by 12% SDS-PAGE and blotted to polyvinylidene difluoride membrane. EH-interacting proteins were detected on the membrane with the Intersectin EHa fused to AP (EHa-AP) and detected with a chemiluminescent substrate (CSPD®). The amount of protein immobilized on the membrane was determined with Ponceau S staining.

shown). Based on these *in vitro* data, we propose that Intersectin interacts with multiple components of the endocytic machinery in a manner to be defined in future experiments.

DISCUSSION

We report the sequence and initial characterization of a novel protein, Intersectin, from frog, which likely serves as a scaffold protein in endocytosis. Intersectin represents the first protein reported with both EH and SH3 domains, two protein interaction modules that recognize NPF and P*XX*P motifs, respectively, in other proteins. Comparison of frog Intersectin
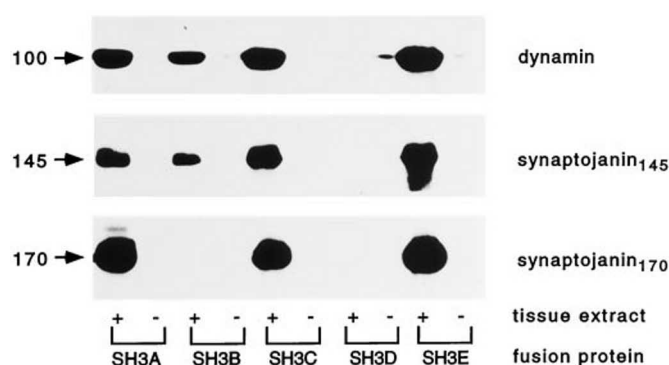
FIG. 8. **The SH3 domains of Intersectin pull-down dynamin and synaptojanin from rat brain extracts.** GST fusion proteins encoding the various SH3 domains of Intersectin (as indicated) were conjugated to glutathione-Sepharose and incubated in the presence (+) or absence (−) of Triton X-100-soluble proteins from adult (*top two panels*) or E18 (*bottom panel*) rat brain. Material specifically bound to the beads was eluted with SDS-PAGE sample buffer. The panels show immunoblots with antibodies against dynamin, synaptojanin$_{145}$, and synaptojanin$_{170}$ as indicated.

to GenBank and The Institute for Genomic Research data bases has identified several cDNA entries, which are extremely similar and are likely members of a gene family. This family consists of DAP160, a protein (36) recently identified in *Drosophila melanogaster*, and human Intersectin, which has recently been discovered in the human genome on chromosome 21.[4]

As a means of understanding the cellular function of Intersectin, we have identified three mouse proteins (*i.e.* RAB/Rip, Ibp1, Ibp2) that bind to the EH domains of Intersectin. Although each of the proteins contains three or four NPF motifs, interestingly, there is a NPF at their C termini. Several lines of evidence suggest that the C-terminal motif is the major site of interaction between these proteins and Intersectin. First, removal of the C-terminal six amino acids of RAB/Rip destroyed binding to Intersectin, whereas grafting the same sequence to GST generated a strong interaction on blots. Second, a computer search of GenBank for other proteins with the same C-terminal sequence successfully predicted that the *Xenopus laevis* mitotic phosphoprotein, MP90 (45), could bind to both EH domains of Intersectin *in vitro*. Third, a synthetic peptide, corresponding to the C-terminal NPF motif of RAB/Rip, competed the interaction between a peptide ligand and the EHa domain 15–25-fold better than peptides corresponding to the internal NPF motifs. Fourth, the ability of the C-terminal peptide to compete in the same assay is 7-fold better when the peptide has a free carboxylate *versus* a carboxamide group. Fifth, the EHa domain only binds to GST fusions bearing the NPF motif when the motif is within two residues of the carboxylate group. Thus, it appears that the free carboxylate group from the C terminus contributes to binding, much like it does for PDZ ligands (55). Because the motif NPFXD has been independently shown to be a endocytosis signal in *Saccharomyces cerevisiae* (56), it will be interesting to learn whether or not acidic amino acids can substitute for the C-terminal carboxylate in peptide ligands for EH domains.

We hypothesize that Intersectin is a new component of the endocytic machinery. Sequence analysis of the three Intersectin interacting proteins (*i.e.* Ibp1, Ipb2, and MP90) has revealed the presence of putative clathrin-binding sites (46–48). In addition, a rat protein, Epsin, which appears to be the homolog of mouse Ibp1, has been cloned very recently and

---

shown to bind to clathrin and its adaptor, AP-2 (57). Thus, it would appear that Intersectin has the potential to bind to clathrin-coated pits or vesicles in cells through its EH domains. Analysis of the SH3 domains of Intersectin has shown that dynamin and synaptojanin are potential interacting proteins as well. Both proteins have large proline-rich domains that have been shown to bind SH3 domain-containing proteins (21, 24) of the endocytic machinery. Independent analysis of the SH3 domains of Dap160 has revealed that they bind dynamin and synaptojanin as well, along with several other novel proteins (36). Furthermore, immunofluorescent detection of Dap160 in neurons has revealed that the protein co-localizes with dynamin in zones of endocytosis at the membrane of the nerve terminus (36).

Although it is possible that the two EH domains of Intersectin interact with different proteins, we hypothesize that the EHa domain, which appears to have stronger binding properties than the EHb domain, binds to the C termini of target proteins and that the EHb domain binds to internal NPF sites. From experiments comparing the binding properties of linear and cyclized NPF-containing peptides, we suspect that the tertiary structure of the protein strongly influences the binding of the internal NPF sites. A bivalent interaction could be quite stable due to avidity effects. Such a multivalent arrangement may be quite common, as most EH domain containing proteins have multiple EH domains, and interacting proteins may contain multiple ligand sites (58). For example, synaptojanin$_{170}$ has three NPF repeats that interact with the EH domains of Eps15, and this interaction may help recruit synaptojanin$_{170}$ to clathrin-coated pits (27, 28). We suspect the interactions of Intersectin with dynamin and synaptojanin may also be based on multivalent interactions, because both proteins have large proline-rich domains. Future experiments will map the sites of interaction and verify that they occur *in vivo*.

REFERENCES

1. Di Fiore, P. P., Pelicci, P. G., and Sorkin, A. (1997) *Trends Biochem. Sci.* **22,** 411–413
2. Wong, W. T., Schumacher, C., Salcini, A. E., Romano, A., Castagnino, P., Pelicci, P. G., and Di Fiore, P. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 9530–9534
3. Carbone, R., Fre, S., Iannolo, G., Belleudi, F., Mancini, P., Pelicci, P. G., Torrisi, M. R., and Di Fiore, P. P. (1997) *Cancer Res.* **57,** 5498–5504
4. van Delft, S., Schumacher, C., Hage, W., Verkleij, A., and van Bergen en Henegouwen, P. (1997) *J. Cell Biol.* **136,** 811–821
5. Cupers, P., Jadhav, A. P., and Kirchhausen, T. (1998) *J. Biol. Chem.* **273,** 1847–1850
6. Salcini, A. E., Confalonieri, S., Doria, M., Santolini, E., Tassi, E., Minenkova, O., Cesareni, G., Pelicci, P. G., and Di Fiore, P. P. (1997) *Genes Dev.* **11,** 2239–2249
7. Benedetti, H., Raths, S., Crausaz, F., and Riezman, H. (1994) *Mol. Biol. Cell* **5,** 1023–1037
8. Tang, H. Y., and Cai, M. (1996) *Mol. Cell. Biol.* **16,** 4897–4914
9. Wendland, B., McCaffery, J. M., Xiao, Q., and Emr, S. D. (1996) *J. Cell Biol.* **135,** 1485–1500
10. Cohen, G. B., Ren, R., and Baltimore, D. (1995) *Cell* **80,** 237–248
11. Pawson, T. (1995) *Nature* **373,** 573–560
12. Ren, R., Mayer, B. J., Cicchetti, P., and Baltimore, D. (1993) *Science* **259,** 1157–1161
13. Sparks, A., Rider, J., Hoffman, N., Fowlkes, D., Quilliam, L., and Kay, B. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 1540–1544
14. Leto, T. L., Adams, A. G., and DeMendez, I. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 10650–10654
15. Pleiman, C. M., Hertz, W. M., and Cambier, J. C. (1994) *Science* **263,** 1609–1612
16. Gout, I., Dhand, R., Hiles, I. D., Fry, M. J., Panayotou, G., Das, P., Truong, O., Totty, N. F., Hsuan, J., Booker, G. W., Campbell, I. D., and Waterfield, M. D. (1993) *Cell* **75,** 25–36
17. Bar-Sagi, D., Rotin, D., Batzer, A., Mandiyan, V., and Schlessinger, J. (1993) *Cell* **74,** 83–91
18. David, C., McPherson, P. S., Mundigl, O., and De Camilli, P. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 331–335
19. McPherson, P. S., Garcia, E. P., Slepnev, V. I., David, C., Zhang, X., Grabs, D., Sossin, W. S., Bauerfeind, R., Nemoto, Y., and De Camilli, P. (1996) *Nature* **379,** 353–357
20. Leprince, C., Romero, F., Cussac, D., Vayssiere, B., Berger, R., Tavitian, A., and Camonis, J. H. (1997) *J. Biol. Chem.* **272,** 15101–15105
21. Ramjaun, A. R., Micheva, K. D., Bouchelet, I., and McPherson, P. S. (1997) *J. Biol. Chem.* **272,** 16700–16706
22. Wigge, P., Kohler, K., Vallis, Y., Doyle, C. A., Owen, D., Hunt, S. P., and McMahon, H. T. (1997) *Mol. Biol. Cell* **8,** 2003–2015
23. de Heuvel, E., Bell, A. W., Ramjaun, A. R., Wong, K., Sossin, W. S., and McPherson, P. S. (1997) *J. Biol. Chem.* **272,** 8710–8716
24. Ringstad, N., Nemoto, Y., and De Camilli, P. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94,** 8569–8574
25. Micheva, K. D., Kay, B. K., and McPherson, P. S. (1997) *J. Biol. Chem.* **272,** 27239–27245
26. Ramjaun, A. R., and McPherson, P. S. (1996) *J. Biol. Chem.* **271,** 24856–24861
27. Haffner, C., Takei, K., Chen, H., Ringstad, N., Hudson, A., Butler, M. H., Salcini, A. E., Di Fiore, P. P., and De Camilli, P. (1997) *FEBS Lett.* **419,** 175–180
28. McPherson, P. S., de Heuvel, E., Phillie, J., Wang, W., Sengar, A., and Egan, S. (1998) *Biochem. Biophys. Res. Commun.* **244,** 701–715
29. Sparks, A. B., Hoffman, N. G., McConnell, S. J., Fowlkes, D. M., and Kay, B. K. (1996) *Nat. Biotech.* **14,** 741–744
30. Short, J. M., Fernadez, J. M., Sorge, J. A., and Huse, W. D. (1988) *Nucleic Acids Res.* **16,** 7583–7600
31. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) *Science* **239,** 487–491
32. Sparks, A., Adey, N., Cwirla, S., and Kay, B. (1996) in *Phage Display of Peptides and Proteins: A Laboratory Manual* (Kay, B. K., Winter, J., and McCafferty, J., eds) pp. 227–253, Academic Press, San Diego
33. Felici, F., Castagnoli, L., Musacchio, A., Jappelli, R., and Cesareni, G. (1991) *J. Mol. Biol.* **222,** 301–310
34. Yamabhai, M., and Kay, B. K. (1997) *Anal. Biochem.* **247,** 143–151
35. McPherson, P. S., Takei, K., Schmid, S. L., and De Camilli, P. (1994) *J. Biol. Chem.* **269,** 30132–30139
36. Roos, J., and Kelly, R. B. (1998) *J. Biol. Chem.* **273,** 19108–19119
37. Cwirla, S. E., Peters, E. A., Barrett, R. W., and Dower, W. J. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6378–6382
38. Devlin, J. J., Panganiban, L., and Devlin, P. E. (1990) *Science* **249,** 404–406
39. Scott, J. K., and Smith, G. P. (1990) *Science* **249,** 386–390
40. Glucksman, M. J., Bhattacharjee, S., and Makowski, L. (1992) *J. Mol. Biol.* **226,** 455–470
41. Kishchenko, G., Batliwala, H., and Makowski, L. (1994) *J. Mol. Biol.* **241,** 208–213
42. Petrenko, V. A., Smith, G. P., Gong, X., and Quinn, T. (1996) *Protein Eng.* **9,** 797–801
43. Bogerd, H. P., Fridell, R. A., Madore, S., and Cullen, B. R. (1995) *Cell* **82,** 485–494
44. Fritz, C. C., Zapp, M. L., and Green, M. R. (1995) *Nature* **376,** 530–533
45. Stukenberg, P. T., Lustig, K. D., McGarry, T. J., King, R. W., Kuang, J., and Kirschner, M. W. (1997) *Curr. Biol.* **7,** 338–348
46. Goodman, O. B., Jr., Krupnick, J. G., Gurevich, V. V., Benovic, J. L., and Keen, J. H. (1997) *J. Biol. Chem.* **272,** 15017–15022
47. Dell'Angelica, E., Klumperman, J., Stoorvogel, W., and Bonifacino, J. (1998) *Science* **280,** 431–434
48. Ramjaun, A. R., and McPherson, P. S. (1998) *J. Neurochem.* **70,** 2369–2376
49. Fazioli, F., Minichiello, L., Matoskova, B., Wong, W. T., and Di Fiore, P. P. (1993) *Mol. Cell. Biol.* **13,** 5814–5828
50. Benmerah, A., Gagnon, J., Begue, B., Megarbane, B., Dautry-Varsat, A., and Cerf-Bensussan, N. (1995) *J. Cell Biol.* **131,** 1831–1838
51. Benmerah, A., Begue, B., Dautry-Varsat, A., and Cerf-Bensussan, N. (1996) *J. Biol. Chem.* **271,** 12111–12116
52. Berger, B., Wilson, D. B., Wolf, E., Tonchev, T., Milla, M., and Kim, P. S. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 8259–8263
53. Jones, H., and Hooley, R. (1997) *Plant Physiol* **113,** 663
54. de Beer, T., Carter, R. E., Lobel-Rice, K. E., Sorkin, A., and Overduin, M. (1998) *Science* **28,** 1357–1360
55. Saras, J., and Heldin, C. H. (1996) *Trends Biochem. Sci.* **21,** 455–458
56. Tan, P. K., Howard, J. P., and Payne, G. S. (1996) *J. Cell Biol.* **135,** 1789–1800
57. Chen, H., Fre, S., Slepnev, V., Capua, M., Takei, K., Butler, M., Di Fiore, P., and De Camilli, P. (1998) *Nature* **394,** 793–798
58. Wendland, B., and Emr, S. D. (1998) *J. Cell Biol.* **141,** 71–84